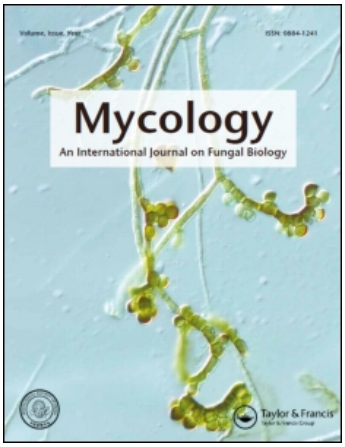

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Effective arbuscular mycorrhizal fungi in the roots of *Tetraclinis articulata* and *Lavandula multifida* in Moroccan *Tetraclinis* woodlands

Salah Eddine Bakkali Yakhlef[a]; Younes Abbas[a]; Yves Prin[b]; Mohamed Abourouh[a]; Marie-Mathilde Perrineau[b]; Robin Duponnois[cd]
[a] Centre de Recherche Forestière, Charia Omar Ibn Khattab, B.P. 763, Agdal-Rabat, Maroc [b] CIRAD, Laboratoire des Symbioses Tropicales et Mèditerranèennes (LSTM), UMR 113 CIRAD/INRA/IRD/SupAgro/UM2, Campus International de Baillarguet, TA A-82/J, Montpellier, France [c] IRD, Laboratoire des Symbioses Tropicales et Mèditerranèennes (LSTM), UMR 113 CIRAD/INRA/IRD/SupAgro/UM2, Campus International de Baillarguet, TA A-82/J, Montpellier, France [d] Laboratoire Ecologie et Environnement (Unité associée au CNRST, URAC 32), Faculté des Sciences Semlalia. Université Cadi Ayyad, Marrakech, Maroc

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Effective arbuscular mycorrhizal fungi in the roots of *Tetraclinis articulata* and *Lavandula multifida* in Moroccan *Tetraclinis* woodlands

Salah Eddine Bakkali Yakhlef[a]*, Younes Abbas[a], Yves Prin[b], Mohamed Abourouh[a], Marie-Mathilde Perrineau[b] and Robin Duponnois[c,d]

[a]*Centre de Recherche Forestière, Charia Omar Ibn Khattab, B.P. 763 Agdal-Rabat, Maroc;* [b]*CIRAD, Laboratoire des Symbioses Tropicales et Mèditerranèennes (LSTM), UMR 113 CIRAD/INRA/IRD/SupAgro/UM2, Campus International de Baillarguet, TA A-82/J, Montpellier, France;* [c]*IRD, Laboratoire des Symbioses Tropicales et Mèditerranèennes (LSTM), UMR 113 CIRAD/INRA/IRD/SupAgro/UM2, Campus International de Baillarguet, TA A-82/J, Montpellier, France;* [d]*Laboratoire Ecologie et Environnement (Unité associée au CNRST, URAC 32), Faculté des Sciences Semlalia. Université Cadi Ayyad, Marrakech. Maroc*

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The diversity of arbuscular mycorrhizal fungi (AMF) in roots of two plant species (arar, *Tetraclinis articulata* and lavender, *Lavandula multifida*) that exist alone or co-occur in Moroccan *Tetraclinis* woodlands was characterized using molecular techniques. The interaction between the two plant species on their root AMF communities was also studied. We analysed the large subunit (LSU) ribosomal RNA gene amplified by nested PCR from a root DNA extract using AM fungal-specific primers. A total of 200 cloned fragments from five root bulk samples of the two plant species were analysed by restriction fragment length polymorphism (RFLP) and 30 of them were sequenced. Phylogenetic analyses demonstrated that these RFLP types correspond to eight phylotypes: five belonged to the Gigasporaceae and three to the Glomeraceae. The highest diversity was found in the roots of *T. articulata* alone, followed by the association *T. articulata*/*L. multifida* and, finally, *L. multifida* alone. Many AMF were shared between the two species, although a few of them had a preference for one or the other. In addition, PCA analysis showed clearly that the AMF community colonizing *T. articulata* differed significantly from that colonizing *L. multifida*. Our results reveal an impact of plant interactions on the AMF community.

Keywords: arbuscular mycorrhizal fungi; diversity; LSU rRNA gene; *Tetraclinis articulata*; *Lavandula multifida*; Morocco

Introduction

Man-mediated degradation, from excessive industrial exploitation, clearing of land for agricultural purposes, and irregularity of rainfall distribution have led to a dramatic regression in Moroccan forest ecosystems, particularly *Tetraclinis* woodlands. Arbuscular mycorrhizal (AM) inoculation has been shown to improve survival of transplants in degraded Mediterranean environments where soil microbial activity has been reduced (Requena 2001). AM symbioses may also provide benefits to plants in terms of nutrient acquisition, maintenance of plant water status and protection from pathogens (Smith and Read 1997). Abbas et al. (2006) reported that Moroccan *Tetraclinis articulata* is an AM-dependent species.

Due to the key ecological functions played by AM symbiosis (Jeffries et al. 2003), management of native soil mycorrhizal potential through drought-tolerant, native and highly mycotrophic plant species is one of the main reclamation strategies to increase revegetation success (Duponnois et al. 2001; Azcon-Aguilar et al. 2003). The mycorrhizal potential contribution of small woody members of the shrubland community, such as *Lavandula multifida* or *Olea oleaster*, frequently observed in *Tetraclinis* woodlands in Morocco, has been assessed; *L. multifida* showed a high mycorrhizal potential number (MPN) (Bakkali et al. 2008). There is evidence that there are selection pressures on both the plant and the fungus to be more specific in their choice of partners (Sanders and Fitter 1992; Bever et al. 1996), although this does not necessarily result in complete specificity, but rather a kind of host preference for certain AMF (Sanders 2002).

The composition of AMF communities plays an important role in plant community structure and diversity (Van der Heijden et al. 1998a, b; O'Connor et al. 2002), so that changes in the AMF community could modify the plant community and, hence, ecosystem processes. However, the influence of the host-plant on an AMF community in plant roots is poorly documented in Morocco. An understanding of this process in Moroccan natural *Tetraclinis* woodland requires knowledge of AMF distribution and their abundance in root systems. A molecular approach is needed to characterize the AM fungal species *in planta* and to focus

*Corresponding author. Email: bakkali_yse@yahoo.fr

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on the actively growing and functional fungi (Helgason et al. 1999; Clapp et al. 2002). Nested PCR based on the large subunit (LSU) region of ribosomal RNA genes (rDNA) has also been successfully used to detect AMF species colonizing plant roots in microcosm experiments (Van Tuinen et al. 1998a; Jacquot et al. 2000; Kjoller and Rosendahl 2000), and to study AMF diversity in the field (Jacquot-Plumey et al. 2001; Turnau et al. 2001).

Several research groups have used group-specific or general primers to recognize rDNA for measurement of AMF diversity in roots (Helgason et al. 1998, 1999; Daniell et al. 2001; Vanderkoornhuyse et al. 2002a,b). Other studies have demonstrated the co-occurrence of several different AMFs within plant root system (Helgason et al. 1998, 1999, 2002; Daniell et al. 2001; Vanderkoornhuyse et al. 2002a,b), indicating some AMF host preference (Helgason et al. 2002; Vanderkoornhuyse et al. 2002a).

The present study addresses three questions: (i) What is the AMF diversity in roots of the *Tetraclinis* woodland plants? (ii) Do co-occurring plant species share a similar AMF community in their roots? (iii) Is the fungal community of coexisting plants change with their interaction? To address these questions, two host species (*T. articulata* and *L. multifida*), which co-occur in the *Tetraclinis* ecosystem, were sampled. Nested PCR was used to describe the diversity and identities of AMF in the co-occurred host species and assess the AMF–host plant preference under different ecological situations.

Materials and methods

Site description and soil analyses

The study was conducted in the *Tetraclinis* woodland of Benslimane, Morocco (altitude: 191 m; coordinates: N 33° 40′ 665″, W 007° 00′ 525″). The climate is medium to highly moderate semiarid and the substratum is schistose or siliceous with relatively sandy schist and pelite. The physical and chemical soil characteristics are: pH: 6.7; clay (%): 12.3; silt (%): 27.70; sand (%): 54.1; organic matter (%): 4.81; total-N (‰): 0.25; assimilable P (mg/100): 1.41. The herbaceous stratum is dominated by *Olea oleaster*, *Phillyrea media*, *Prasium majus*, *Arisarum vulgare*, *Cistus monspeliensis*, *C. salviifolius*, *C. albidus*, *Lavandula multifida*, *L. stoechas*, and *Asphodelus microcarpus*.

Root sampling

Five adult plants of each species: *T. articulata* alone (T), *T. articulata* associated to *L. multifida* (T_l), *L. multifida* alone (L) and finally *L. multifida* associated with *T. articulata* (L_t), were randomly selected. Roots of each species were pooled to represent one sample; they were harvested by removing soil blocks close to the base of these plants. In the laboratory, the collected roots were thoroughly washed under running tap water to remove attached soil and debris. Root samples were kept at –20 °C in 1.5-ml reaction tubes for subsequent molecular analysis.

DNA extraction from roots colonized by AMF

Total DNA was extracted from 150 mg of mycorrhizal root samples using the DNeasy Plant Mini Kit (Qiagen, France) following the manufacturer's instructions. A nested PCR approach was used to amplify the root extract DNA. The first PCR was performed with the eukaryotic-specific primer LR1 [5′-GCA TAT CAA TAA GCG GAG GA-3′] (Van Tuinen et al. 1998b) and with the fungal-specific primer FLR2 [5′-GTC GTT TAA AGC CAT TAC GTC-3′] (Trouvelot et al. 1998). Reactions were performed in a final volume of 20 μl containing 10× PCR buffer (Promega, Madison, WI, USA), 200 nM dNTPs, 500 nM each primer and 0.4 U Taq polymerase (Promega). A 1-μl aliquot of diluted DNA extract was added to 19 μl of the PCR mix. The PCR program was as follows: initial denaturation at 93°C (1 min), 93°C for 30 s, 58°C for 1 min and 72°C for 1 min (35 cycles), followed by 10 min at 72°C. PCR products were diluted 1/100 and used as templates for the second PCR with the primers FLR3 [5′-TTG AAA GGG AAA CGA TTG AAG T-3′] and FLR4 [5′-TAC GTC AAC ATC CTT AAC GAA-3′] under the same PCR conditions. PCR products were run on a 2% agarose gel in TAE buffer and visualised under UV light after staining with ethidium bromide.

Construction of LSU rDNA libraries and sequencing

The PCR products generated from roots of *T. articulata* and *L. multifida* using the primers FLR3 and FLR4 were ligated into the pGEMT vector, which has 3′-T overhangs to facilitate cloning of PCR products (Promega). Ligation mixtures were transformed into ultracompetent *E.coli* XL1-Blue (Stratagene, Cambridge, UK) according to the manufacturer's instructions. From each sample LSU rDNA gene library, putative transformants were screened by PCR using the M13 forward and reverse primers with the conditions described above. A total of 200 positive clones, 50 from each LSU rDNA gene library, were subjected to RFLP analysis with *Alu*I and *Hinf* I. PCR products were re-amplified from representatives of each RFLP type using M13 forward/M13 reverse, cleaned using Qiaquick PCR purification spin columns (Qiagen Ltd, Crawley, UK) and sequenced.

Reconstruction of phylogenetic tree

Both strands of 30 cloned DNA fragments were sequenced separately using the BigDye Terminator Cycle Sequencing Kit, the AmpliTaq DNA Polymerase FS

(Perkin Elmer Applied Biosystems, Foster, City, CA, USA) and the M13 forward (-20) primer. Sequencing products were analyzed using the automated ABI PRISM 310 DNA Genetic Analyzer (Perkin Elmer Applied Biosystems) at the DNA Sequencing Facilities of INRA (Nancy, France). The sequencing data were edited using Sequencher (Genes Codes Corporation, Ann Arbor, MI, USA) for Macintosh computers. A search for chimeric sequences was performed using the RDP Chimera Check Program. A total of five sequences was clearly chimeric and was excluded from our further analysis. Sequences were compared using the BlastN program at the national Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/BLAST/index) (Altschul et al. 1997). The sequences will be deposited at the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis used the maximum likelihood method implemented in PhyML program at the www.phylogeny.fr site (Dereeper et al. 2008). Sequence of *Mortieriella polycephala* (AF113464) was used as out group. Different phylotypes were defined as groups of closely related sequences, usually with a high level of bootstrap support in the phylogenetic analyses (higher than 80%) and of pairwise similarity (higher than 97%).

Diversity index and statistical analysis

As indicators of host specificity and the impact of plant interactions on the diversity of their AMF community, we examined the phylotype's specific richness (*S*) and the Shannon biodiversity index (*H*) (Shannon and Weiner 1963):

$$H = -\Sigma(ni/N)\ln(ni/N)$$

where ni represents the number of sequences belonging to each phylotype and *N* the total number of phylotypes.

We applied a χ^2 test of independence to infer whether or not the composition of the AM fungal communities differed between the two study cases of each target plant. To combine the information provided by the different phylotypes in relation to target plants, a multivariate approach based on Principal Component Analysis (PCA) was performed. All statistical analyses were carried out on SPSS 11.5 for Windows.

Results

RFLP-typing and phylogenetic analysis

The amplification product obtained from mycorrhizal root DNA after the first PCR amplification with the primer pair LR1–FLR2 was about 750 bp. The second PCR, using taxon-specific primers (FLR3–FLR4) and the first amplification products as template DNA, discriminated between *Glomales* species in all mycorrhizal roots. The sizes of the amplification products by the nested PCR protocol varied between 300 and 380 bp. An example of the amplification products obtained after nested PCR with the specific primers is presented in Figure 1. Similar nested PCR types were obtained from roots of *T. articulata* and *L. multifida*. This shows that at least two different AMF colonize the four different mycorrhizal root sample types. FLR3–FLR4 PCR products were used to construct LSU rDNA libraries from roots of *T. articulata* and *L. multifida*. Fifty positive clones from each library were subjected to RFLP analysis to select the main specific types to be sequenced. Thirteen different RFLP patterns were identified. The RFLP type's percentage varied significantly among the two target species library, being lowest in *L. multifida* (54%) and highest in *T. articulata* (85%). Depending on the RFLP type frequencies, up to five cloned DNA fragments of each RFLP type were sequenced. The distribution of the AMF RFLP types in the different plant species is shown in Table 1. Sequence analyses showed that the 25 sequences had a high similarity to known LSU rDNA sequences from AMF. The phylogenetic analysis revealed eight AMF sequence types or phylotypes: five belonged to Gigasporaceae (GIGA1, GIGA2, SCUT1, SCUT2 and SCUT3) and three to the Glomeraceae (GLOM1, GLOM2 and GLOM3) (Table 1, Figure 2). Adhering to the classification scheme proposed herein, the RFLP types recovered from roots of the two target plants alone or in association belonged almost equally to the two AMF families. Using the selected restriction enzymes, different RFLP patterns may be shared by one phylotype (Table 1). The first most common type (RFLP3, 24%) represents a sequence variant that is not closely related to current reference strains as other, e.g. RFLP8 (Figure 2). The two most common RFLP types (RFLP2, RFLP4), comprising 17.5 and 12.5%, were shared by GLOM2 phylotype. RFLP patterns were approximations for delineating phylotype. Therefore, the more

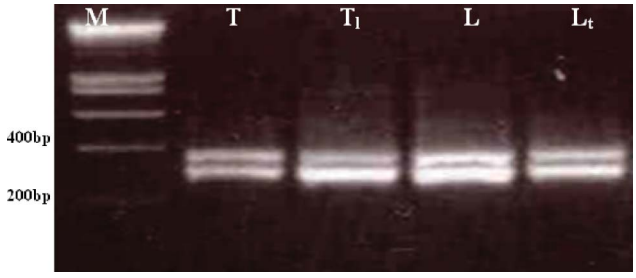

Figure 1. Nested PCR products, with LR1-FLR2 and FLR3-FLR4 primers pair, obtained using DNA extracted from roots. T, T_1: roots of *T. articulata*/respectively alone and in association with *L. multifida*. L, L_t: roots of *L. multifida* respectively alone and in association with *T. articulata*. (M: 100-bp ladder).

Table 1. Phylotype, sequence type and abundance of RFLP pattern clones obtained from each library root. Numbers with asterix represent chimeric sequences.

RFLP pattern	Sequence type	Target plants (Number of clones)	Phylotype
1	Seq 01-1, 2, 3, 4*, 5	T(8) T_l(8) L(12) L_t(10)	GLO1
2	Seq 02-1, 2, 3, 4	T(8) T_l(11) L(10) L_t(6)	GLO2
3	Seq 03	T(13) T_l(8) L(10) L_t(17)	GIG1
4	Seq 04-1, 2*	T(3) T_l(8) L(7) L_t(7)	GLO2
5	Seq 05	L(6)	GLO1
6	Seq 06-1, 2*	L_t(5)	SCU3
7	Seq 07-1, 3, 4, 5	T(4) L(5) L_t(5)	SCU2
8	Seq 08	T(4)	GLO3
9	Seq 09-1, 2*	T_l(5)	GLO1
10	Seq 10	T(3)	GLO1
11	Seq 11-1, 2, 3, 4	T(5) T_l(3)	SCU1
12	Seq 12	T(2) T_l(4)	GIG2
13	Seq 13-1, 2*	T_l(3)	GLO1

rigorous maximum likelihood (ML) approach was used for further analyses.

Host preference and impact of plant interaction on the AMF community

The detected AMF phylotype communities colonizing roots of the target plants in the four studied cases are shown in Figure 3. Richness and diversity indices based on the eight AMF phylotypes were calculated for each plant. Two trends were observed when comparing the target species alone or in association. First, the overall fungal diversity and species richness were higher in T (Shannon diversity index, $H = 2.17$; richness, $S = 7$) and lowest in L ($H = 1.19$, $S = 4$), and AMF communities differed significantly ($\chi^2 = 14.48$, df $= 6$, $p = 0.025$). Second, there was no difference between species richness ($S = 5$) in L_t and T_l with, respectively, a diversity index of 1.61 and 1.41, whereas the composition of their AMF communities differed significantly ($\chi^2 = 22.75$, df $= 6$, $p = 0.001$). These results indicate a host preference impact. Of the eight phylotypes identified, GLOM2, GLOM1 and GIGA1 were dominant, representing, respectively, 30, 27.5 and 24% of the observed sequences (Figure 3). These phylotypes occurred in all plant species. GLOM3, GIGA2, SCUT1, SCUT2 and SCUT3 phylotypes constituted less than 19% of the clones. Phylotypes GLOM3 and SCUT3 were only present in T and L_t, respectively (Figure 3).

The composition of the AMF communities colonizing each target plant, arar and lavender (*T. articulata* and *L. multifida*), at the two situation, alone and in association, differed significantly ($\chi^2 = 13.41$, df $= 6$, $p = 0.037$; $\chi^2 = 9.63$, df $= 4$, $p = 0.047$, respectively). In addition, the AMF communities were also compared using PCA, an independent data analysis that plots the AMF community from each root sample type in a multidimensional space. This analysis showed that *T. articulata* and *L. multifida*, alone and/or in association, are distant from each other (Figure 4), indicating that they harboured a distinct AMF community. Principal component 1 and 2 accounted for most of the variance (52 and 34% for PC1 and PC2, respectively). These results showed also that the colonizing AMF community is not only influenced by host plants but also by their interaction.

Discussion

Mycorrhizal symbiosis is a key component in helping plants to establish in degraded soils. In Mediterranean ecosystems, the use of drought-tolerant, native plant species has been proposed to accelerate natural successions and to re-establish functional shrublands (Francis and Thornes 1990; Herrera et al. 1993). Arid and semi-arid ecosystems are generally characterised by a patchy distribution of individual plant species (Halvorson et al. 1994). The vegetation patches commonly create "fertility islands" (Garner and Steinberger 1989), or "resource islands" (Schlesinger et al. 1996). Therefore, before initiating a *T. articulata* revegetation program, it is necessary to study the existing vegetation and its associated mycorrhizal propagules (Requena 1996). In Moroccan *Tetraclinis* woodlands, *L. multifida* has a patchy distribution and the two plant species are mycorrhizal-dependent (Azcon and Barea 1997; Ouahmane et al. 2006; Abbas et al. 2006). Carillo-Garcia et al. (1999) suggest that this shrub species could be used as a 'plant nurse' for Mediterranean woodland sustainability. In this context, it has been necessary to evaluate the AMF preference of the two target plants.

Analysis of AMF spores isolated from soils may not necessarily reflect the diversity of AMF colonising roots of individual plants species (Clapp et al. 1995; Kjoller and Rosendahl 2001). The difficulty in identifying AMF

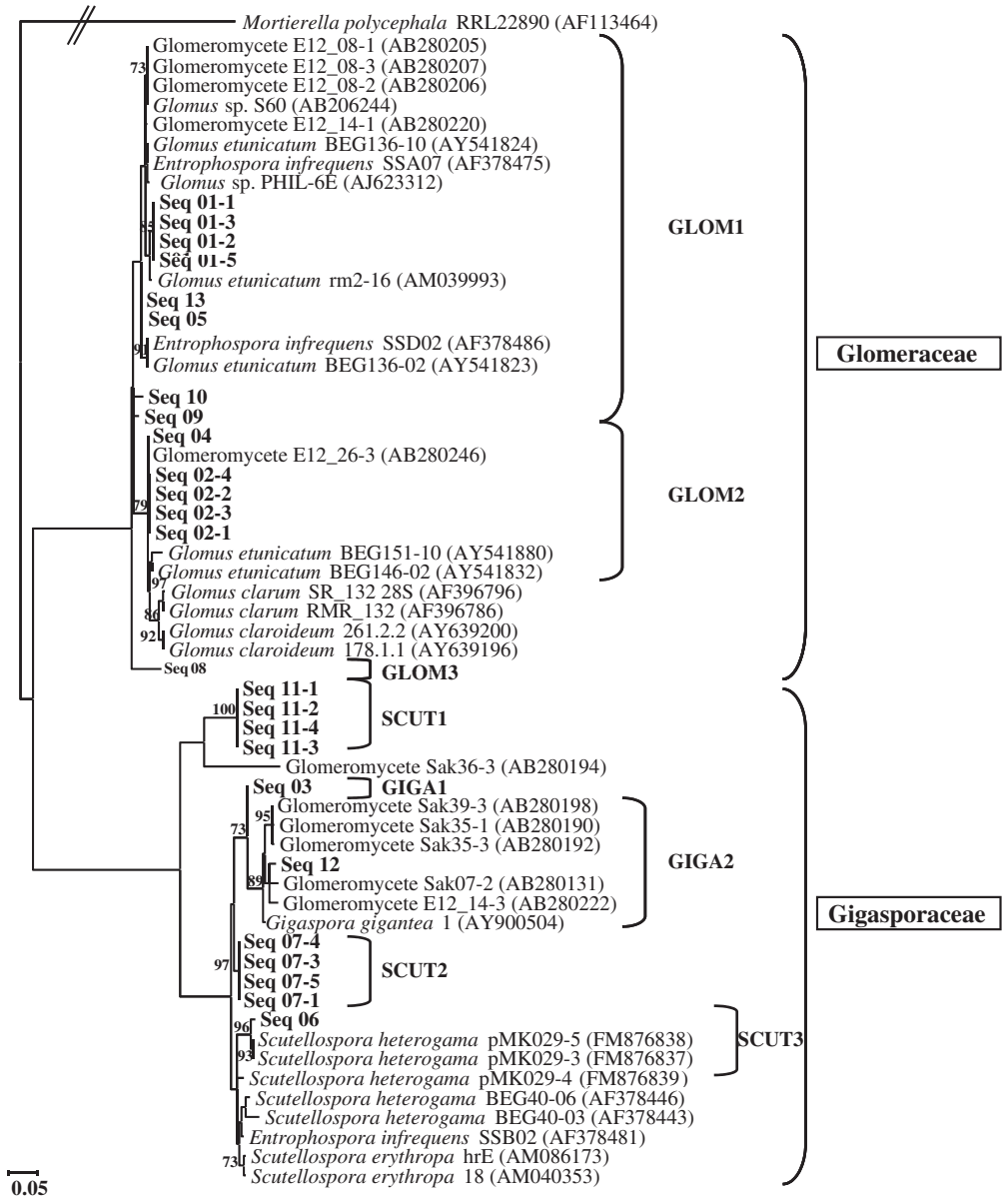

Figure 2. Maximum likelihood phylogenetic tree of arbuscular mycorrhizal (AMF) colonizing roots of *T. articulata* and *L. multifida*. *Mortierella polycephala* was used as an outgroup. Bootstrap values calculated from 500 replicates (using phyML) are shown at tree nodes, only for values over 70%.

in plant roots has always been an obstacle to the study of their community dynamics in ecosystems. Morphological discrimination may be possible at the genus level when hyphal morphology is sufficiently different between fungi (Merryweather and Fitter 1998). Molecular techniques have been developed to discriminate AMF coexisting within root plants. Nested PCR offers several advantages over these different approaches for monitoring the behaviour of a microcosm fungal community *in planta* and soil (Van Tuinen et al. 1998a; Gollotte et al. 2004; Jacquot et al. 2000; Pivato et al. 2007). In the present study, nested PCR of the LSU rDNA was successfully applied to characterize the AMF diversity in roots.

This study is the first to describe the AMF colonizing roots of *T. articulata* and *L. multifida* alone or in association in Moroccan *Tetraclinis* woodland and to evaluate their interaction within communities. The genetic diversity of the AMF in roots of the target plants was characterized by analysing 50 clones of each LSU rDNA library. The number of clones analysed was in the same range as the numbers used in previous diversity studies (Vandenkoornhuyse et al. 2002a; Gollotte et al. 2004). Based on RFLP analysis,

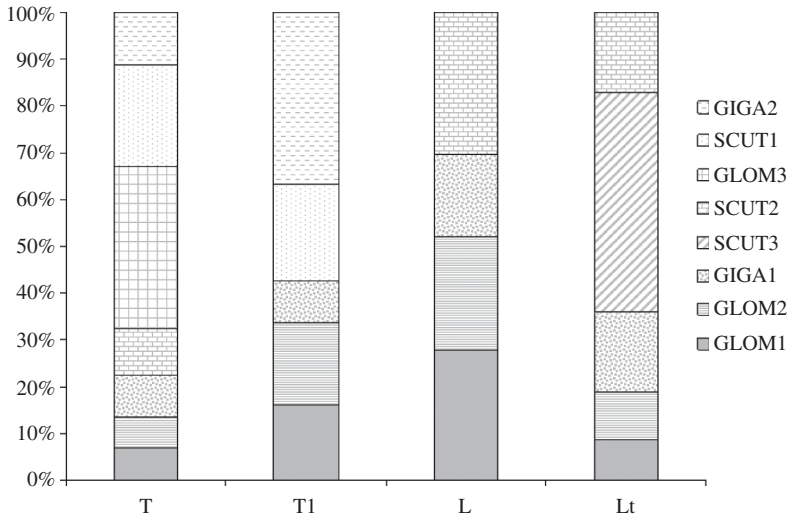

Figure 3. AMF communities found in the roots of *T. articulata* and *L. multifida*. T, T_l: roots of *T. articulata* respectively alone and in association with *L. multifida*. L, L_t: roots of *L. multifida* respectively alone and in association with *T. articulata*.

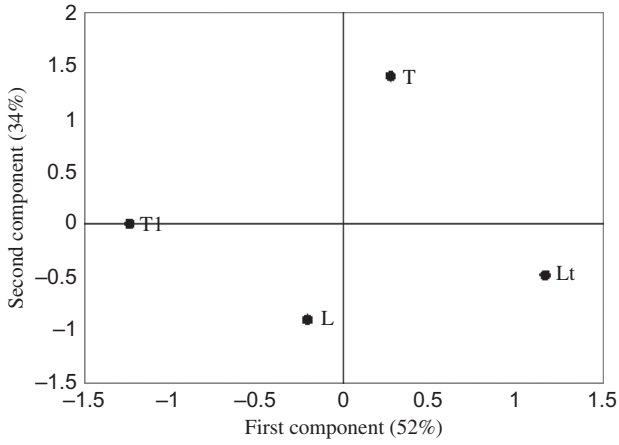

Figure 4. Plot of factor scores, along Principal Components axes 1 and 2, for the AMF communities found in the roots of *T. articulata* and *L. multifida*. T, T_l: roots of *T. articulata* respectively alone and in association with *L. multifida*. L, L_t: roots of *L. multifida* respectively alone and in association with *T. articulata*. Numbers in brackets represent the percentages of variance explained by the PC.

we have found 13 different AMF types in the roots of the two plant species. The phylotypes were defined on the basis of the bootstrap level (>80%) after a phylogenetic analysis. The phylogenetic tree including known sequences showed that all AMF sequences collected from roots of *T. articulata* and *L. multifida*, alone or in association, belong to two families, the Glomeraceae and Gigasporaceae. The dominance of the genus *Glomus* is not new. Predominance of Glomeraceae species is in accordance with previous report based on morphological characters of Moroccan *Tetraclinis* woodland spores (Abbas et al. 2006). Daniell et al. (2001) argued that *Glomus* types dominate the colonization of arable crops because *Glomus* species are better adapted to disturbed environments. One possible reason why *Glomus* species are dominant in a disturbed ecosystem may be found in the ability of the Glomeraceae to colonize via fragments of mycelium or mycorrhizal roots pieces (Biermann and Linderman 1983). In our study, we reported also a predominance of Gigasporaceae, which propagate only via spores (Daniell et al. 2001). We suggest that, in our study site, the two families may share similar roots infectivity.

Furthermore, the AMF diversity indices were measured for each root plant in the presence or in the absence of the other plant. The level of diversity as indicated by Shannon diversity indices was very high in *T. articulata* compared to *L. multifida*. This could be explained by the age differences and the nature of the plants. It has been established that the Glomalean fungi invade the seedling's single root, and colonization intensity increases as plants age and bulbs descend through the soil profile to soil horizons where P is highly deficient and mycorrhiza are

essential (Merryweather and Fitter 1995). However, the similar diversity indices in root of the neighbouring target plants suggest the impact of the plant interaction on the AMF community. There is evidence that there are selection pressure on both the plant and the fungus to be more specific in their choice of partners (Sanders et al. 1992; Bever et al. 1996).

These results are supported by the PCA plot, which shows how in different ecological situations plant species harbour different AMF communities. This agrees with previous studies that have reported some host preference in AMF (Helgason et al. 2002; Vandenkoornhuyse et al. 2002b 2003). Moreover, many AMF species were shared between the two target plants, indicating a similar AMF affinity. This may support the hypothesis that *L. multifida* could be used as a 'plant nurse' on further *T. articulata* revegetation programmes in Morocco. Ouahmane et al. (2006) reported that *Lavandula* spp. act as potential 'plant nurses' for the early growth of Moroccan *Cupressus atlantica*. In our field site, a large number of sequences must be analysed for identifying both AMF host preference and affinity in natural ecosystem.

In conclusion, we have shown that the mycorrhizal community colonizing roots of two co-existing plants in *Tetraclinis* woodland is highly diverse. Particular plant–fungus combinations may be favoured according to ecological conditions. The molecular evaluation of AMF populations within roots is, therefore, a promising approach for identifying naturally occurring fungi which efficiently colonize plant roots in field sites, such as Mediterranean soil ecosystems. Effective AMF could be isolated and selected as inocula for the revegetation of such stressed environments with appropriate plant species.

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